

Genotoxicity-suppressing effect of *Sophora japonica* L. aqueous extract

ABSTRACT

The dry flower buds of *Sophora japonica* L. are used as a hemostatic agent in traditional Chinese medicine. In the comet assay, aqueous extracts of *S. japonica* decreased and increased the tail length significantly in cultured human lymphoblastoid WTK1 cells exposed to UV in the absence and presence of DNA repair inhibitors, respectively. The extract did not affect the tail length in methyl methanesulfonate-exposed cells. In the present study, the aqueous extract of the flower buds of *S. japonica* was separated by repeated column chromatography, yielding four types of flavonoid glycosides. Among them, only rutin, similar to the extract, decreased and increased the tail length significantly in WTK1 cells exposed to UV in the absence and presence of DNA repair inhibitors, respectively.

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The genotoxicity-suppressing effect of rutin was further studied using the micronucleus test. Rutin significantly decreased the frequency of micronucleated binucleate cells in UV-exposed WTK1 cells but did not affect this frequency in UV-exposed XPL3KA (Xeroderma pigmentosum group C) cells. These results suggest that the anti-genotoxic potential of rutin is due to an enhanced incision step of global genome repair (GGR) sub-pathways in nucleotide excision repair (NER). Herein, we show that *S. japonica* exhibits heretofore unknown anti-genotoxic potential against UV by enhancing the incision of GGR sub-pathways in NER, and that its anti-genotoxic component is rutin.

Keywords: *Sophora japonica* L. extract, rutin, anti-genotoxic potential, incision step, nucleotide excision repair.

1. INTRODUCTION

The dry flower buds of *Sophora japonica* L. are used as a hemostatic agent in traditional Chinese medicine. These buds contain high levels of rutin [1–3] and are a commercial source of the compound [4]; they have also been found to contain various flavonoid glycosides. Some components of foods and medicinal plants, e.g., tea catechins and iridoids, are to show genotoxicity-suppressing effects [5–7]. In general, catechins play an important role in the anti-mutagenic of tea [5, 8]. In this study, we investigated the unknown anti-mutagenic effects of the flavonoid glycosides isolated from the aqueous extract of *S. japonica* flower buds.

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2. MATERIAL AND METHODS

2.1 General Experimental Procedures

All NMR spectra were recorded on a JEOL instrument operating at 400 MHz for ¹H, using standard pulse sequences. Chemical shifts are were reported on the δ scale in parts per million, downfield from TMS. Column chromatography was performed on Silica gel 60 (Merck), a highly porous polymer (HP-20, Alfa Aesar). TLC was carried out on precoated Silica gel 60 F254 plates (Merck) developed with EtOAc-n-hexane, CHCl₃-MeOH, and n-BuOH-AcOH-H₂O.

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2.2 Extraction and Isolation

Dried and powdered pericarps of *S. japonica* (300 g) were extracted with boiling water for 40 min and cooled to room temperature. The precipitate (NK1) was filtered, dissolved in MeOH, concentrated, and then subjected to column chromatography using a highly porous polymer eluted with the gradient solutions H₂O and MeOH (0–60%), yielding seven fractions. Fraction 7 was subjected to column chromatography using silica gel eluted with CHCl₃-MeOH-H₂O (7:3:0.5), yielding six fractions. Fraction 7-4 (NK2) was crystallized from EtOAc-MeOH and filtered to afford rutin as a yellow powder (1, 265 mg). Fraction 7-6 was subjected to column chromatography using silica gel eluted with CHCl₃-MeOH-H₂O (9:1:0.05), yielding six fractions. Fraction 7-6-2 was subjected to column chromatography using silica gel eluted with the gradient solutions EtOAc and n-hexane (50–100%), yielding four fractions. Each fraction was purified using HPLC (ODS column eluted with the gradient solutions MeOH and H₂O (10–90%)), affording prunetin (2, 47.2 mg) (fraction 7-6-2-2, NK3), isorhamnetin 3-O-rutinoside (3, 53.1 mg) (fraction 7-6-2-3, NK4), and genistein 7,4'-di-O-β-D-glucopyranoside (4, 59.5 mg) (fraction 7-6-2-4, NK5). The fractionation methods used is as Fraction method is summarized in Fig. 1.

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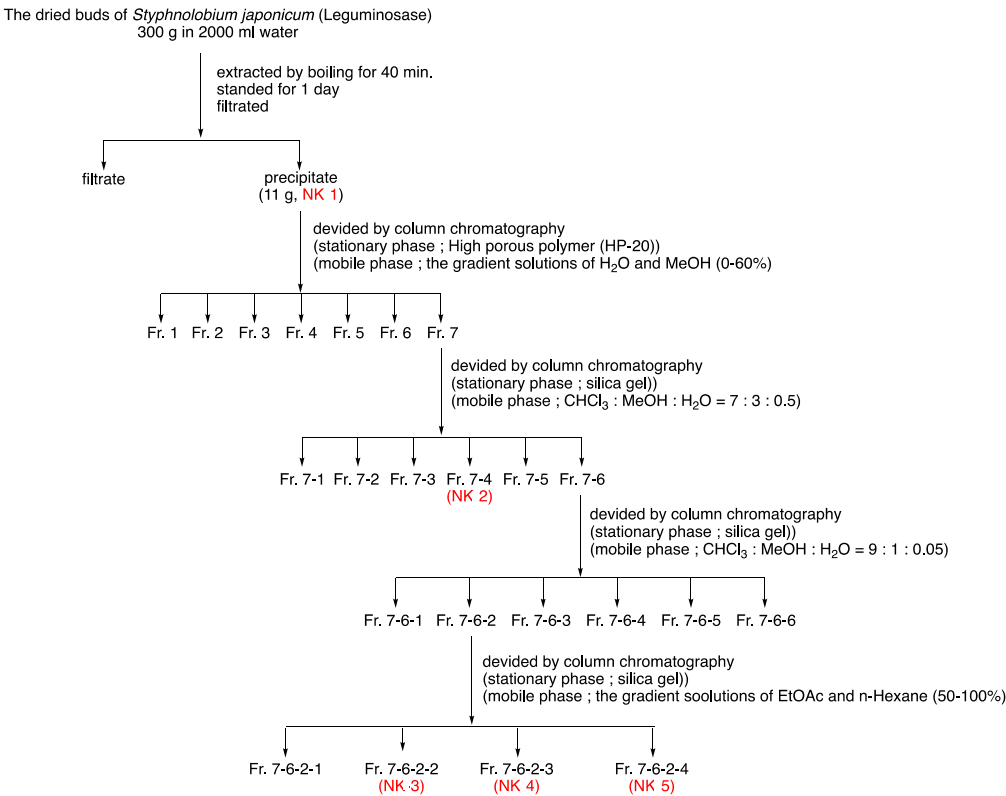


Fig. 1. Flowchart of fractionation of *S. japonica* aqueous extract

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2.3 Cells

TK^{+/+} heterozygotes of WTK1 human lymphoblastoid cells exhibiting wild-type p53 (kindly provided by Dr. Honma, National Institute of Health Sciences, Tokyo) and XPL3KA Epstein Barr virus-transformed human lymphoblastoids (XPCs;

Xeroderma pigmentosum group C; obtained from Health Science Research Resource Bank, Osaka) were used. WTK1 cells were maintained in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd.) supplemented with 10% horse serum (SAFC Biosciences), 200 µg/mL sodium pyruvate, and 200 µg/mL streptomycin at 37 °C under a 5% CO₂ atmosphere. XPCs were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (SAFC Biosciences), 200 µg/mL sodium pyruvate, and 200 µg/mL streptomycin. Cells at a concentration of 5×10^5 cells/mL were exposed to methyl methanesulfonate (MMS) for 2 h and washed twice with Hanks' BSS. For UVC irradiation, 1 mL of cell suspension in saline (5×10^5 cells/mL) in a 6 cm dish was irradiated with a germicide lamp (National GL15, 15 W, Matsushita Electric Industrial Co., Japan) at 25 µW/cm².

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2.4 Comet assay

Cells exposed to MMS or UV were post-treated with each fraction dissolved in dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries, Ltd.) for 2 h in the presence or absence of the DNA repair inhibitors hydroxyurea (HU, 10 mM) and cytosine-1-β-D-arabinofuranoside (araC, 1.8 mM). Both inhibitors (Wako Pure Chemical Industries, Ltd.) were dissolved in DMSO. Treated cells were suspended in 1% agarose-LGT at 5×10^5 cells/75 µL, and 75 µL of cell suspension was immediately deposited on a fully frosted slide (Matsunami Glass Ind., Ltd., Osaka, Japan), which was coated with 1% agarose GP-42 and then covered with another glass slide. The slides were placed so as to allow the agarose to gel. The samples on the slides were then immediately exposed to a lysing solution (pH 10) of 2.5 M NaCl, 100 mM EDTA disodium (Na₂EDTA), 10 mM Trizma, 1% sarkosyl, 10% DMSO, and 1% Triton X-100, and left at 4 °C for 1 h. The slides were then placed on a horizontal gel electrophoresis platform and covered with pH >13 alkaline solution composed of 300 mM NaOH and 1 mM Na₂EDTA. The slides were left in solution at 0 °C for 20 min to allow unwinding of the DNA and expression of alkali-labile sites. The power supply was set at 25 V and 250 mA. The DNA was subjected to electrophoresis at 0 °C for 20 min and the slides were rinsed with 400 mM Trizma (pH 7.5) to neutralize the excess alkalinity. Each slide was stained with 50 µL of 20 µg/mL ethidium bromide (Wako Pure Chemical Industries, Ltd.) and covered with a cover slip. Fifty cells on one slide per dose (one slide was prepared for each dose) were examined and photographed (black and white ASA 400 Fuji film) at 200× magnification using a fluorescence microscope (Olympus) equipped with a G filter. The tail length of the comet was measured. The effect of chemical treatment on tail length was analyzed using ANOVA and the Dunnett test.

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2.5 Micronucleus test

Cells exposed to UVC were post-treated with NK2 for 24 h in a fresh medium with cytochalasin B at 3 µg/mL, and then sampled.

The collected cells were suspended in 0.075 M KCl hypotonic solution for 15 min; the cell suspension was concentrated to a volume of 1 mL, mixed with 1 mL of 10% neutral buffered formalin solution, and then concentrated to a volume of 100 µL. The cell suspension was further mixed with 100 µL of 0.05 w/v% aqueous solution of acridine orange, and then 50 µL of the mixture was placed onto a slide glass and mounted with 24 × 48 mm cover slips. Micronucleated binucleate cells (MNBNCs) per 1000 binucleate cells (BNCs) were scored with the aid of a fluorescence microscope (Olympus at 600× magnification) equipped with a B filter. Relative BNC (BNCs at each concentration of NK2 compared with that of an untreated control) numbers were obtained. The effect of chemical treatment on MNBNCs was analyzed using ANOVA and the Dunnett test.

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3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Chemical analysis Spectra characterization and identification of compounds in *S. japonica* L. aqueous extract

NK2 Rutin (1). Yellow powder, ¹H-NMR (400 MHz, CD₃OD) δ 7.56 (1H, dd, J = 9.0, 2.1 Hz), 7.55 (1H, d, J = 2.1 Hz), 6.85 (1H, d, J = 8.4 Hz), 6.40 (1H, d, J = 2.0 Hz), 6.11 (1H, d, J = 2.2 Hz), 5.35 (1H, d, J = 7.8 Hz), 5.12 (1H, d, J = 1.6 Hz), 3.83–3.35 (10H, m), 1.13 (3H, d, J = 6.2 Hz).

NK3 Prunetin (2). Yellow powder, ¹H NMR (400 MHz, CD₃OD) δ 8.38 (1H, s), 7.37 (2H, d, J = 8.5 Hz), 6.78 (2H, d, J = 8.5 Hz), 6.66 (1H, d, J = 2.1 Hz), 6.41 (1H, d, J = 2.2 Hz), 3.68 (3H, s).

NK4 Isorhamnetin-3-O-rutinoside (**3**). Yellow amorphous, ^1H NMR (400 MHz, CD_3OD) δ 8.00 (1H, d, $J = 2.0$ Hz), 7.66 (1H, dd, $J = 8.5$ Hz, 2.0 Hz), 6.95 (1H, d, $J = 8.5$ Hz), 6.46 (1H, d, $J = 2.0$ Hz), 6.25 (1H, d, $J = 2.0$ Hz), 5.30 (1H, d, $J = 7.4$ Hz), 4.57 (1H, d, $J = 1.4$ Hz), 4.00 (3H, s), 3.87–3.25 (10H, m), 1.13 (3H, d, $J = 6.2$ Hz).

NK5 Genistein 7,4-di-O- β -D-glucopyranoside (**4**). Yellow amorphous, ^1H NMR (400 MHz, CD_3OD) δ 8.48 (1H, s), 7.52 (2H, d, $J = 8.5$ Hz), 7.12 (2H, d, $J = 8.5$ Hz), 6.73 (1H, s), 6.48 (1H, s), 5.34 (1H, d, $J = 7.4$ Hz), 4.90–4.61 (5H, m), 3.26 (2H, m), 3.47–3.14 (10H, m).

Identified flavonoid glycosides were shown in Fig. 2.

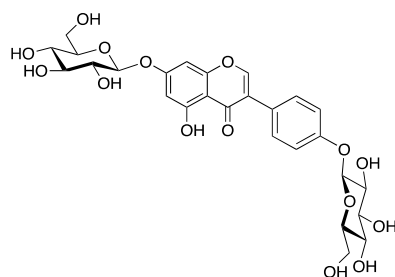
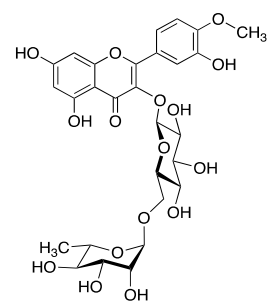
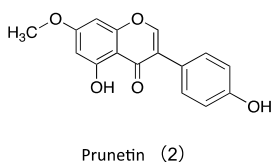
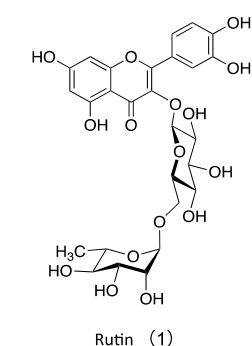


Fig. 2. Identified flavonoid glycosides

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3.2 Comet assay

In UV exposed WTK1 cells, the crude extract decreased and increased tail length significantly in the absence and presence of DNA repair inhibitors (araC/HU), respectively (Fig. 3). Relative survival was >70% with and without DNA

araC/HU, showing that observed decrease and increase in tail length were not due to cytotoxic effect of the extract. On the other hand, it did not affect tail length in MMS exposed WTK1 cells (Fig. 3). In UV exposed WTK1 cells, NK2 similar to the crude extract, increased and decreased tail length significantly with and without DNA repair inhibitors, respectively (Fig. 4). Relative survival was >70% with and without DNA repair inhibitors, showing that observed decrease and increase in tail length were not due to cytotoxic effect of NK2. Neither NK3, NK4, nor NK5 did not affect tail length with and without DNA repair inhibitors. The crude extract and all studied fractions did not affect tail length in mutagen-unexposed WTK1 cells, suggesting that they do not have genotoxic potential in WTK1 cells.

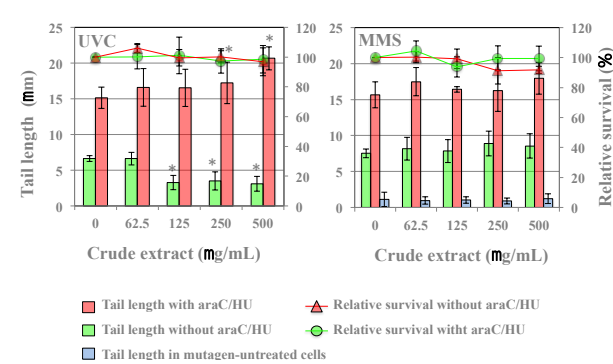


Fig. 3. Effects of post-treatment with crude extract of *S. japonica* on tail length in WTK1 cells.

Cells exposed to UV radiation or MMS were post-treated with a crude extract of *S. japonica* and its fractions for 2 h. The error bars indicate standard errors of the mean. Significantly different from untreated control, * $P < 0.05$; $Mean \pm S.D = Mean\ values \pm standard\ deviation\ of\ three\ independent\ trials$.

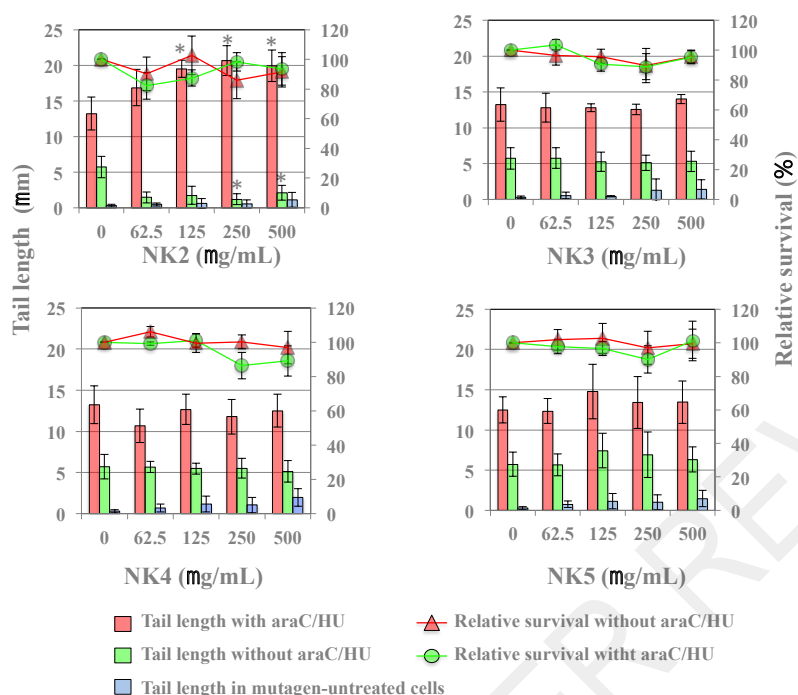


Fig 4. Effects of post-treatment with fractions of *S. japonica* extract on tail length in WTK1 cells.

Cells exposed to UV radiation or MMS were post-treated with fractions of *S. japonica* for 2 h. The error bars indicate standard errors of the mean. Significantly different from untreated control, * $P < 0.05$; Mean \pm S.D = Mean values \pm standard deviation of three independent trials.

3.3 Micronucleus test

The effect of NK2 was further studied by the micronucleus test. NK2 decreased MNBNC significantly in UV-exposed WTK1 cells but not in UV-exposed XPL3KA (Fig. 5). Because NK2 did not cause BNC frequency suppression in relative BNC at $<1000 \mu\text{g/mL}$, the observed decreases in MNBNC frequency was not due to cell cycle delay caused by cytotoxicity. NK2 did not affect BNC frequency in UV-unexposed WTK1 cells in WTK1 and XPL3KA cells, showing that NK2 was not considered to be a direct clastogen in both cells.

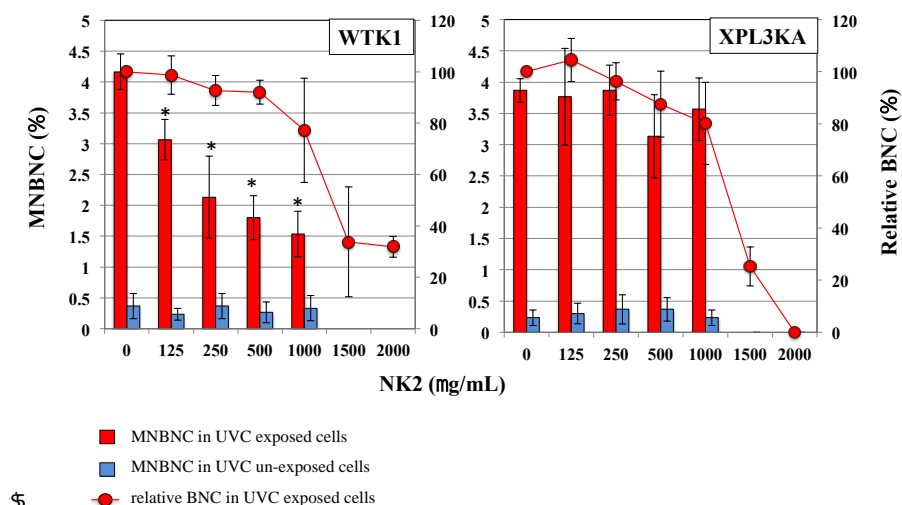


Fig. 5. Effects of post-treatment with NK2 on frequency of MNBNCs in WTK1 and XPL3KA cells.

Cells exposed to UV radiation or MMS were post-treated with NK2 for 24 h. The error bars indicate standard errors of the mean. Significantly different from untreated control, * $P < 0.05$; Mean \pm S.D. = Mean values \pm standard deviation of three independent trials.

4. Discussion

Four types of flavonoid glycosides were isolated from the aqueous extract of the flower buds of *S. japonica*. Since out of 4 fractions only NK2 showed anti-genotoxic potential and NK2 was identified to be rutin, we elucidated that the one of these isolated flavonoid glycosides, rutin would have anti-genotoxic potential.

Some anti-genotoxic chemicals inactivate mutagens by chemical or enzymatic interaction before they attack genes and some suppress the process of mutation fixation after genes are damaged [12]. The induction of micronuclei by UV was suppressed by post-treatment with NK2 (rutin). Since there was no opportunity for inactivation of mutagens by chemical, physical or enzymatic interaction in the post-treatment schedule, NK2 (rutin) should suppress the process of micronucleus formation after DNA lesion is produced. Xeroderma pigmentosum (XP) is an autosomal recessive human genetic disorder characterized by extreme sensitivity to sunlight, a high incidence of skin cancer on sun-exposed skin and neurological complications [10]. XP consists of eight different genetic complementation groups, groups A through G and a variant (XP-A through XP-G and XP-V). The primary defect in XP-A through XP-G, with the exception of XP-V, which has a defect in translesion DNA synthesis, resides in an early stage of nucleotide excision repair (NER). There are two sub-pathways in NER [14]. One is designated as transcription-coupled DNA repair (TCR), which preferentially occurs in the transcribed strand of transcriptionally active genes. The other is global genome repair (GGR), which occurs throughout the genome including the non-transcribed strand of active genes. Both NER sub-pathways are defective in groups A through G, except group C in which only GGR is impaired [12]. NK2 (rutin) was shown not to affect the frequency of MNBNC in UV exposed GGR-defective XPL3KA cells, that is, in XP-C, which would suggest that NK2 (rutin) affects GGR sub-pathways in NER to show its anti-genotoxicity potential against UV. We previously showed that single strand break (SSB) formation through the incision step of NER followed by SSB rejoining was accelerated by wild-type *p53* [13]. Considering that rutin showed anti-genotoxicity potential in WTK1 cells having mutant *p53*, however, its anti-genotoxicity should not be due to *p53* status.

The induction of comet tail by UV was suppressed by the post-treatment with NK2 (rutin) in the absence of DNA repair inhibitors. The comet assay detects single strand breaks (SSBs) produced as initial lesions and also those that are

generated during the repair of initial lesions such as alkylated bases, bulky base adducts and pyrimidine dimers [14]. Pyrimidine dimers are well known to be induced by UV. In the case of UV, comet assay-detectable SSBs and/or alkali-labile sites are formed during the excision repair process [14]. It is known that pyrimidine dimers are repaired by NER, which consists of the following four steps: recognition of the DNA lesion, excision of a 24–32 nucleotide stretch containing the lesion by dual incision of the damaged DNA strand on both sides, filling in of the resulting gap by DNA polymerase and ligation of the nick. During the process, SSBs are produced as intermediates, which can be visualized as comet tail in the comet assay. Therefore, if NK2 (rutin) inhibits the incision step of NER, SSB formation would be reduced. If so, the observed results would suggest co-genotoxicity but not anti-genotoxicity, which seems to contradict to micronucleus suppression. DNA breaks formed from DNA lesions, such as pyrimidine dimers, can be accumulated by preventing DNA resynthesis; the combination of HU and araC is often for this purpose [13,15,16]. Comet tail induction by UV was increased by the post-treatment with rutin in the presence of DNA repair inhibitors, which might suggest that SSB formation during excision repair is enhanced in the presence of NK2 (rutin). Therefore, it should be considered that rutin enhances the incision of GGR sub-pathways in NER (where SSBs are formed), which followed by SSB rejoining during NER are enhanced in the presence of rutin, which results in the observed reduction of comet tail and micronucleus formation. Although there are no data on rutin in MMS exposed cells, the crude extract had no effect on MMS, suggesting that its component, rutin, also has no effect on MMS, which is consistent with the findings that rutin does not have anti-genotoxic potential against oxidative stress by increasing the rate of DNA repair [17]. Although photoproducts upon UV exposure and bulky adducts upon exposure to a multitude of chemicals are removed by NER [18 - 20], oxidized, methylated, and deaminated bases are removed by base excision repair (BER) [21]. Considering this knowledge, if rutin enhances the incision of GGR sub-pathways in NER, it would be well explained the reason why rutin shows anti-genotoxic effect against UV but not MMS and oxidative stress.

4. CONCLUSION

In conclusion, we showed here unknown anti-genotoxic potential of *S. japonica* against UV by enhancing the incision of GGR sub-pathways in NER. To our knowledge, this is the first report that shows *S. japonica* and its component, rutin, have anti-genotoxic potential against UV by enhancing the capacity of NER.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS DISCLAIMER:

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